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GRANT NUMBER DAMD17-97-1-7105

TITLE: (alpha)2(beta)1 Integrin-Induced Breast Cancer
Differentiation

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REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Aug 97 - 31 Jul 98)	
4. TITLE AND SUBTITLE (alpha)2(beta)1 Integrin-Induced Breast Cancer Differentiation				5. FUNDING NUMBERS DAMD17-97-1-7105	
6. AUTHOR(S) Kamata, Tetsuji, M.D., Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Scripps Research Institute La Jolla, California 92037				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				<div style="border: 1px solid black; padding: 10px; display: inline-block;"> 19990614 061 </div>	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) <p>Cell-extracellular matrix (ECM) interaction is important for cell differentiation in mammary epithelial cells. This cell-ECM interaction is mediated by integrins, a family of cell adhesion receptors that mediate cell-cell and cell-ECM interactions, and play a critical role in cell migration, proliferation, and differentiation. The integrin $\alpha 2 \beta 1$ functions as a collagen and/or laminin receptor. Previous reports suggest that $\alpha 2 \beta 1$ plays a critical role in normal mammary cell differentiation as well as in the pathogenesis of breast cancer. Therefore, $\alpha 2 \beta 1$-ligand interaction can be a key factor in controlling breast cancer differentiation. The purpose of this study is to clarify the role of $\alpha 2 \beta 1$-ligand interaction in breast cancer differentiation, and to elucidate the molecular mechanism of $\alpha 2 \beta 1$-ligand interaction. During the first year of this project, I found that (1) ligand binding-defective mutant $\alpha 2$ can be stably expressed in a poorly differentiated mammary carcinoma cell line, Mm5MT, and (2) the collagen-contact sites in $\alpha 2 \beta 1$ are localized in the three separate loop structures surrounding the MIDAS (metal ion dependent adhesion site) motif in the I(A) domain of $\alpha 2$. These findings will form the basis for the remainder of this study.</p>					
14. SUBJECT TERMS Breast Cancer Integrin, Collagen, Laminin				15. NUMBER OF PAGES 16	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

FOREWORD

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Tetsuji Kamada 8/27/88
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TABLE OF CONTENTS

Front Cover	1
Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	7
Experimental Methods and Procedures	7
Mutagenesis	7
Cell culture and transfection	7
Adhesion assay	8
Results and Discussion	8
Wild-type and mutant $\alpha 2$ are surface- Expressed in Mm5MT	8
Discontinuous multiple loops in the I Domain are required for collagen binding	8
Recommendations in Relation to the Statement of Work	9
Conclusions	10
References	10
Legends to the figures	13
Figure 1	14
Figure 2	15
Figure 3	16

INTRODUCTION

Cell-extracellular matrix (ECM) interaction has a profound impact on cell structure and function (1). There is overwhelming evidence that the ECM influences mammary epithelial cell differentiation (2). When mammary epithelial cells are cultured in reconstituted basement membrane gels, they undergo morphogenesis and exhibit many of the characteristics of alveoli *in vivo* (3). Cell-ECM interactions are altered in malignant neoplastic cells. The loss of normal cell-ECM interaction is associated with a loss of cellular differentiation and a loss of adhesion-dependent regulation of cell growth (4). Cell-ECM interaction is mediated through a variety of cell surface receptors including integrins, which play a major role in cell-ECM interactions. Integrins are a supergene family of adhesion receptors that consist of at least 15 α and 8 β subunits (5-9). Each integrin molecule is composed of structurally different α and β subunits and functions as a receptor for extracellular matrix proteins or cell surface ligands. Structurally, each α or β subunit consists of a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The ligand binding specificity of each integrin is determined by its combination of α and β subunits. Many integrins have overlapping binding specificities. For example, the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ both bind to collagen and laminin. Besides binding to the ECM and cellular ligands, integrins contribute to the regulation of cell shape, morphology, and motility by assembling cytoskeletal proteins through cytoplasmic domains. Also, integrins activate a variety of signal transduction pathways(10).

What role do integrins play in mammary differentiation? Mammary cells acquire a glandular morphology and express mammary-specific genes when cultured in Matrigel(3, 11). The first clue to suggest the role of integrins in these processes comes from a paper by Streuli et al(2) in which they report that β -casein expression in mammary epithelial cells requires $\beta 1$ integrins. Another clue comes from a paper on the analysis of $\alpha 2\beta 1$ expression in normal and neoplastic carcinoma tissues(12) in which it is reported that, while normal mammary epithelial cells express high-level $\alpha 2\beta 1$ integrin, neoplastic mammary tissue expresses less $\alpha 2\beta 1$ as it becomes less differentiated. Berdichevsky et al. showed that collagen-induced morphogenesis of mammary epithelial cells in a monolayer culture could be inhibited by anti- $\alpha 2$ or anti- $\beta 1$ monoclonal antibodies(13). This same group also showed that the

expression of *c-erbB2* in mammary epithelial cells causes a decrease in $\alpha 2\beta 1$ expression and impairs the cells' ability to undergo morphogenesis in a collagen gel(14). More direct evidence of $\alpha 2\beta 1$ participation in mammary cell differentiation comes from a paper by Zutter et al(15) in which they report that the expression of $\alpha 2\beta 1$ integrin in poorly differentiated mammary carcinoma cells resulted in a phenotypic alteration from fibroblastoid, non-contact-inhibited, invasive cells to epithelioid, contact-inhibited, less invasive cells. Expression of $\alpha 2\beta 1$ also restored the cells' ability to differentiate into gland-like structures in three-dimensional matrices, and markedly reduced their tumorigenicity. The same group also reported that the reduction of $\alpha 2\beta 1$ expression in well differentiated mammary carcinoma cells resulted in the loss of morphogenesis in collagen gels(16). These reports clearly indicate that $\alpha 2\beta 1$ is critically involved in the differentiation of normal mammary cells as well as that of mammary carcinoma cells. However, the molecular mechanism that leads to differentiation is still not clear. Is $\alpha 2\beta 1$ -collagen interaction required, or is the expression of $\alpha 2\beta 1$ itself enough for differentiation to take place? If $\alpha 2\beta 1$ -collagen interaction is required, what is the role of the $\alpha 2\beta 1$ cytoplasmic domains? Are the $\alpha 2\beta 1$ cytoplasmic domains specifically required? And if the $\alpha 2\beta 1$ cytoplasmic domains are required, what kind of signaling molecules are specifically involved in this process? I intend to answer these questions by expressing ligand-binding defective $\alpha 2$ ($\alpha 2$ that has mutations in its cytoplasmic domains) in a poorly differentiated mammary carcinoma cell line. Finding the answers to these questions will bring us closer to developing therapeutic interventions against breast cancer.

Modulation of $\alpha 2\beta 1$ -ECM interaction could affect mammary cell differentiation (13). Therefore, elucidation of $\alpha 2\beta 1$ -ligand binding mechanisms may lead to the development of therapeutic interventions against breast cancer. $\alpha 2\beta 1$ integrin is a receptor for collagen and laminin. The ligand binding site(s) in $\alpha 2\beta 1$ has been localized in the I (A) domain of the $\alpha 2$ subunit (17, 18). Although several amino acid residues that constitute the metal ion-dependent adhesion site (MIDAS) are critical for ligand binding (19), the specific contact site for each ligand is not well characterized. Based upon the crystal structure of the $\alpha 2$ I domain (20), I will try to identify the specific ligand binding site for collagen or laminin using domain-

swapping mutagenesis and site-directed mutagenesis. Once the specific mutations that block either collagen or laminin are determined, I will express these mutations in a breast cancer cell line and examine their effect on differentiation. The information obtained in this study may form the basis for the development of therapeutic interventions against breast cancer.

BODY

Experimental Methods and Procedures

Mutagenesis

Site-directed mutagenesis was carried out by unique restriction enzyme site elimination with a double stranded vector (21). Loop-swapping mutagenesis was carried out using the overlap extension polymerase chain reaction (22). The amino acid residues in $\alpha 2$ that were replaced with the corresponding αL residues are 152-157 (L βA - $\alpha 1$), 212-219 (L $\alpha 3$ - $\alpha 4$), and 257-262 (L βD - $\alpha 5$). In del αC , residues 283-290 including the C-helix are deleted from $\alpha 2$. The entire I domain (residues 140-367) is deleted from $\alpha 2$ in del I.

Cell culture and transfection

The Murine poorly differentiated mammary carcinoma cell line Mm5MT was purchased from the American Tissue Culture Collection (ATCC). Fifty μg of wild-type or mutant $\alpha 2$ cDNA was transfected into Mm5MT by electroporation. Forty-eight hours after transfection, cells were harvested and surface-expression of $\alpha 2$ was examined by FACS analysis. For stable transfection, 50 μg of wild-type or mutant $\alpha 2$ cDNA was transfected together with 2 μg of pFneo containing a neomycin resistant gene. After transfection, cells were grown in selection medium containing 300 $\mu g/ml$ G418. After 3 weeks, the resulting colonies were harvested with trypsin-EDTA and surface-expression of $\alpha 2$ was examined by FACS analysis.

Transfection of wild-type and mutant $\alpha 2$ into CHO-K1 cells was done as described in (18).

Adhesion Assay

Adhesion of CHO cells transfected with $\alpha 2$ to immobilized collagen type I was quantitated as previously described (18).

Results and Discussion

Wild-type and mutant $\alpha 2$ are surface-expressed in Mm5MT

We previously reported that mutation of Asp-151, Thr-221, and Asp-254 to Ala (D151A, T221A, and D254A, respectively) in $\alpha 2$ completely abolished $\alpha 2\beta 1$ binding to collagen (17, 18). Mutant $\alpha 2$ cDNA subcloned into the mammalian expression vector pBJ-1 was transfected into the mouse mammary carcinoma cell line Mm5MT by electroporation. This cell line lacks endogenous $\alpha 2\beta 1$ integrin and is known to undergo morphogenesis after $\alpha 2\beta 1$ transfection. Fig.1 shows the expression of wild-type and mutant $\alpha 2$ in Mm5MT after transient transfection. All mutants showed expression comparable to that of wild-type (approximately 8.9-14.7% of cells express $\alpha 2$ on the cell surface). I also obtained Mm5MT stably expressing $\alpha 2$ mutants (data not shown). Although the proposed project did not progress as expected due to the difficulty in transfection, these results indicate the feasibility of the current project.

Discontinuous multiple loops in the I domain are required for collagen binding

Amino acid residues that constitute the MIDAS motif, e.g. Asp-151, Thr-221, and Asp-254 in $\alpha 2$, are critical for both divalent cation binding and ligand binding (17, 18). These residues are well conserved among different integrin I domains irrespective of their ligand binding specificities (19). These results suggest that there are amino acid residues that allow specific ligands to bind to particular integrins. By replacing short segments of the $\alpha 2$ I domain with the homologous residues of the αL I domain, which has different ligand binding specificities, and examining the effect on ligand binding, we will be able to identify the specific segments/amino acid residues in $\alpha 2$ that are required for collagen/laminin binding. Based on the crystal structure of the $\alpha 2$ I domain, I replaced loops surrounding the MIDAS motif with the homologous loops of αL (Fig.2). The βE - $\alpha 6$ loop contains an extra C-helix that can be seen only in collagen-binding integrin I domains. This C-helix is believed to create a groove on top of the MIDAS face in which a collagen triple helix docks (20). I created a deletion mutant which deletes most of the

β E- α 6 loop including the C-helix. Fig. 3 shows the result of adhesion of clonal CHO cells expressing mutant α 2 to immobilized collagen type I. The L β A- α 1 and L α 3- α 4 mutants almost completely abolished collagen adhesion even though the MIDAS motif is maintained. The L β D- α 5 mutant partially affected collagen adhesion. Conversely, the del α C mutant did not have any impact on collagen adhesion, even though α C is predicted to have a collagen contact site. These results suggest that the collagen contact sites are localized in the β A- α 1, α 3- α 4, and β D- α 5 loops of the α 2 I domain, and that the C-helix does not contain any critical collagen contact sites. These results are consistent with the fact that the von Willebrand factor A3 domain that does not contain a C-helix also binds to collagen (23). I introduced multiple alanine point mutations within the residues in the β A- α 1, α 3- α 4, β D- α 5, and β E- α 6 loops to identify specific residues that are required for collagen binding. Preliminary results suggest that Ileu-156 and Tyr-157 in β A- α 1; Gln-215, Gly-217, and Gly-218 in α 3- α 4; and Gly-254 in β D- α 5 are critical for collagen binding, even though mutations in the amino acid residues in the β E- α 6 loop did not affect collagen binding (data not shown).

Recommendations in Relation to the Statement of Work

I encountered an unexpected difficulty in transfecting α 2 cDNAs into the mammary carcinoma cell line Mm5MT that caused a delay in this project. While a low transfection efficiency could be overcome by increasing the amount of cDNA used and the electroporation voltage, reliably obtaining a stably transfected cell line is still a problem. Although the current method is working, I intend to try other selection markers and vectors to improve transfection stability and efficiency.

Although the identification of collagen/laminin binding sites in the α 2 I domain was not included in the original proposal, this information may be needed to manipulate mammary cell differentiation. The specific residues required for laminin binding can be identified by using laminin as an adhesion substrate. Once the binding site(s) for collagen and laminin are identified, the specific roles of collagen and laminin in breast cancer cell differentiation can be determined using the proposed assay.

CONCLUSIONS

- 1) Mutant $\alpha 2$ can be stably expressed in a poorly differentiated mammary carcinoma cell line that lacks endogenous $\alpha 2\beta 1$. I will be able to use this system to determine the role of $\alpha 2\beta 1$ in breast cancer differentiation as stated in the original proposal.
- 2) The $\alpha 2\beta 1$ -collagen contact face has been localized in the 3 separate loop structures surrounding the MIDAS motif. The $\alpha 2\beta 1$ -laminin binding face will be determined using the same methodology. The separate roles of collagen and laminin in $\alpha 2\beta 1$ -induced breast cancer differentiation can be clearly determined by using mutants that specifically blocks either collagen or laminin binding.

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Legends to the figures

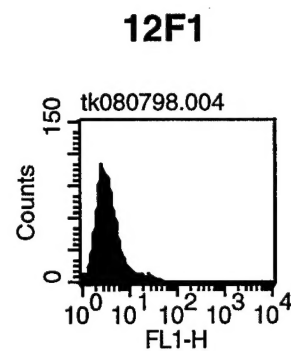
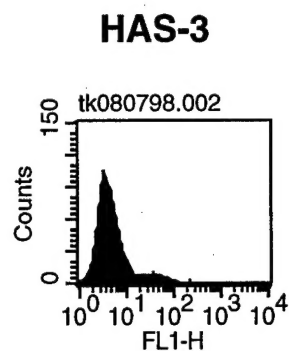
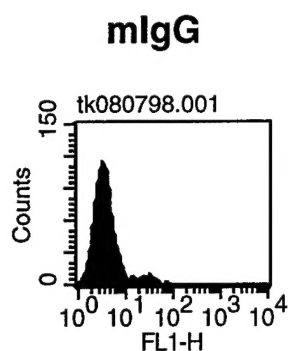
Fig.1. Surface-expression of wild-type and mutant $\alpha 2$ in Mm5MT. Forty-eight hours after transfection, cells were harvested and incubated with mouse IgG, HAS-3 (anti- $\alpha 2$ N-terminal), or 12F1 (anti- $\alpha 2$ I domain), followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. Stained cells were analyzed by flowcytometry. Percent $\alpha 2$ expression as calculated by HAS-3 positivity are 4.36, 14.71, 9.99, 9.83, 8.90, 13.99 for Mm5MT, wild-type, D151A, T221A, D254A, del I, respectively.

Fig. 2. Alignments of human $\alpha 2$ and αL I domains. The β strands and α helices of the $\alpha 2$ I domain are underlined. Swapped regions of $\alpha 2$ and αL (L βA - $\alpha 1$: 152-157; L $\alpha 3$ - $\alpha 4$: 213-219; L βD - $\alpha 5$: 257-262) are outlined in the box. Deleted region in $\alpha 2$ (del αC) is also outlined.

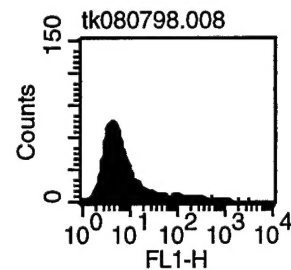
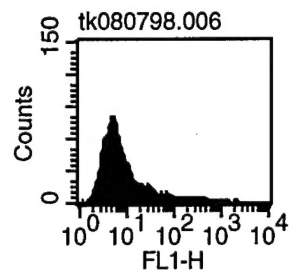
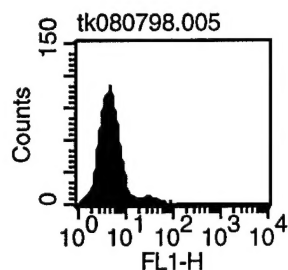
Fig. 3. Adhesion of the mutant $\alpha 2$ -expressing CHO cells to collagen. Clonal CHO cells stably expressing wild-type or mutant $\alpha 2$ were added to the wells coated with BSA (\square), collagen type I (\blacksquare), or fibronectin (\boxtimes). After 1 hour at 37 C, non-adherent cells were removed and the adherent cells were quantitated by endogenous phosphatase assay.

Figure 1

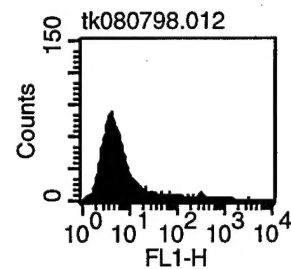
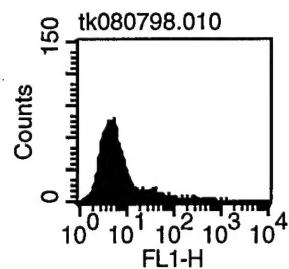
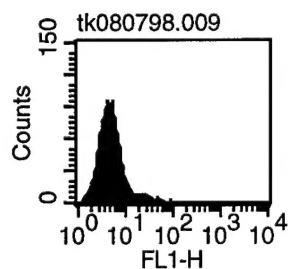
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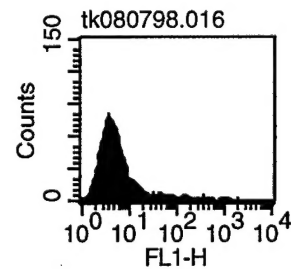
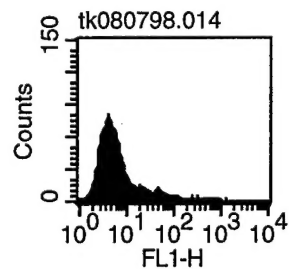
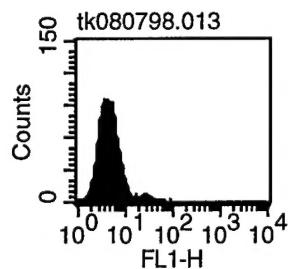
$\alpha 2$ wt



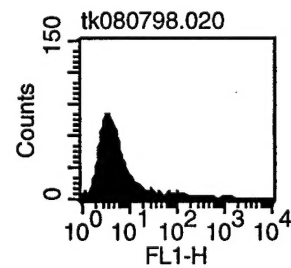
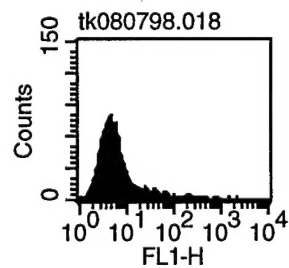
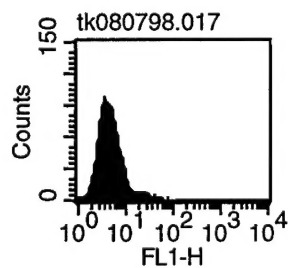
$\alpha 2$ D151A



$\alpha 2$ T221A



$\alpha 2$ D254A



$\alpha 2$ del I

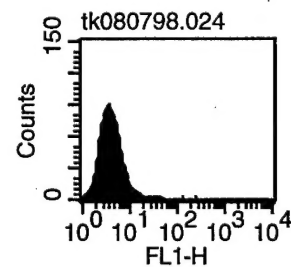
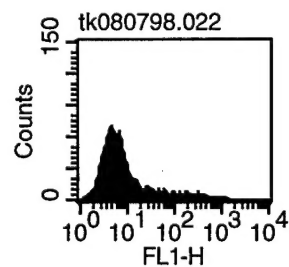
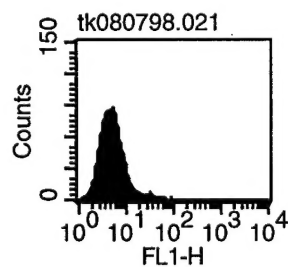


Figure 2

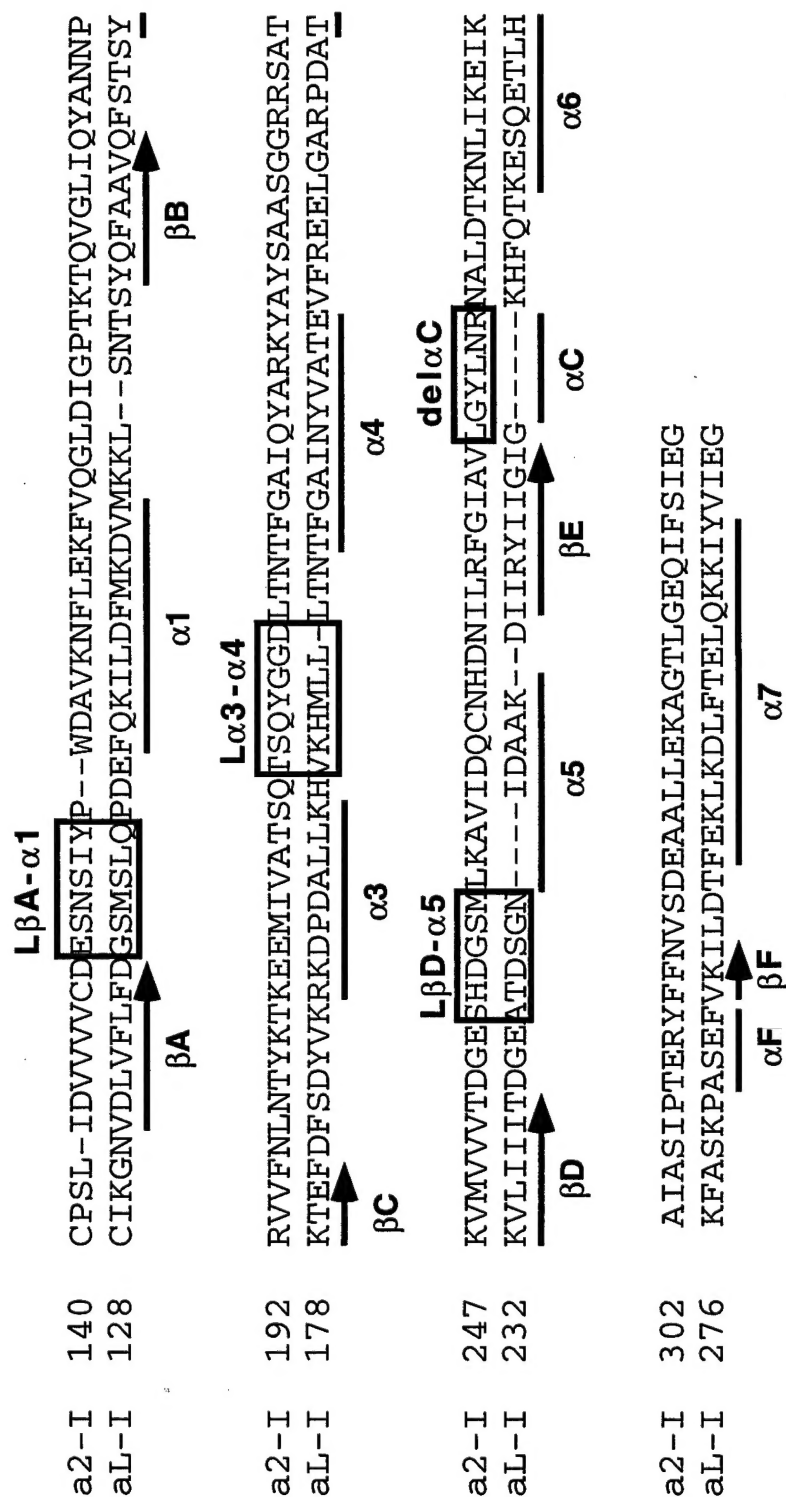


Figure 3

